

University of Groningen

Role of fetoplacental exosomes in fetoplacental endothelial dysfunction in gestational diabetes mellitus

Sáez Gutiérrez, Tamara Andrea

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version

Publisher's PDF, also known as Version of record

Publication date:
2017

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Sáez Gutiérrez, T. A. (2017). *Role of fetoplacental exosomes in fetoplacental endothelial dysfunction in gestational diabetes mellitus*. [Thesis fully internal (DIV), University of Groningen]. University of Groningen.

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

FETOPLACENTAL ENDOTHELIAL EXOSOMES
MODULATE HIGH D-GLUCOSE-INDUCED
ENDOTHELIAL DYSFUNCTION

CHAPTER

3

Submitted

3

Tamara Sáez^{1,2}, Paul de Vos¹, Jeroen Kuipers³, Luis Sobrevia^{2,4,5}, Marijke M Faas^{1,6}

¹Immunoendocrinology, Division of Medical Biology, Department of Pathology and Medical Biology, University of Groningen, University Medical Center Groningen, Hanzeplein 1, 9713 GZ, Groningen, The Netherlands. ²Cellular and Molecular Physiology Laboratory (CMPL), Division of Obstetrics and Gynecology, School of Medicine, Faculty of Medicine, Pontificia Universidad Católica de Chile, Santiago 8330024, Chile. ³Molecular Imaging and Electron Microscopy, Dept Cell Biology, University of Groningen, University Medical Centre Groningen (UMCG), Hanzeplein 1, 9713 GZ, Groningen, The Netherlands. ⁴Department of Physiology, Faculty of Pharmacy, Universidad de Sevilla, Seville E-41012, Spain. ⁵University of Queensland Centre for Clinical Research (UQCCR), Faculty of Medicine and Biomedical Sciences, University of Queensland, Herston, QLD 4029, Queensland, Australia. ⁶Department of Obstetrics and Gynecology, University of Groningen, University Medical Center Groningen, Hanzeplein 1, 9713 GZ, Groningen, The Netherlands.

3

ABSTRACT

Gestational diabetes mellitus (GDM) is associated with fetoplacental endothelial dysfunction. This may be induced by the high levels of glucose in the fetal circulation that influence exosome composition impacting endothelial cell function. To test this, exosomes were isolated from human umbilical vein endothelial cells incubated with basal glucose (5.5 mmol/L; HUVEC- basal glucose ;exo- basal glucose and from HUVECs incubated with high D-glucose (25 mmol/L; HUVEC-HG; exo-HG). HUVEC-basal glucose and HUVEC-high glucose were exposed to exo- basal glucose and exo- high glucose. The effect of exosomes on endothelial cell function was determined by a wound-healing assay and the expression of endothelial nitric oxide synthase (eNOS), human cationic amino acid transporter type 1 (hCAT-1), vascular endothelial growth factor (VEGF) and intracellular adhesion molecule type 1 (ICAM-1) by western blotting, qPCR or flow cytometry. High glucose increased the exosomal release from HUVECs and increased endothelial migration and expression of phosphorylated (P~Ser¹¹⁷⁷) eNOS, hCAT-1, VEGF and ICAM-1. Exo-high glucose also increased endothelial cell migration and P~Ser¹¹⁷⁷-eNOS, and hCAT-1 and ICAM-1 expression in HUVEC- basal glucose. Exo-basal glucose reverted the effect of high glucose on endothelial cell migration and hCAT-1 mRNA expression to normal values. Our results suggest that high glucose induced a GDM-like phenotype in HUVECs and that endothelial exosomes from HUVEC-high glucose could mimic the effects of high glucose on HUVECs. This study contributes to the unraveling of the mechanism by which hyperglycemia affects the fetoplacental vasculature in GDM.

1. INTRODUCTION

Gestational diabetes mellitus (GDM) is one of the most common pregnancy complications in the world [1], affecting 2-6% of all pregnancies in Europe [2]. It is associated with several fetal and maternal complications such as an increased risk for preeclampsia, preterm delivery and fetal macrosomia [3,4]. Moreover, GDM is associated with long-lasting health consequences for both mother and child, such as a chance to develop type 2 diabetes in later life [5–7].

Key in the complications associated with GDM is probably vascular dysfunction in both the maternal and fetoplacental endothelium [1,8]. In human umbilical vein endothelial cells (HUVECs) from women diagnosed with GDM, an impaired endothelial function has been demonstrated. Various changes have for instance been found in the L-Arginine/nitric oxide (NO) signaling pathway in HUVECs derived from GDM pregnancies, such as increased expression of human cationic amino acid transporter type 1 (hCAT-1), increased NO production and increased phosphorylated Ser¹¹⁷⁷ endothelial nitric oxide synthase (P~Ser¹¹⁷⁷eNOS) [9,10], as compared to HUVEC from healthy pregnancies.

However, the mechanisms behind the GDM induced fetal-placental endothelial dysfunction are largely unknown. Since GDM is characterized by maternal and fetal hyperglycemia [1,11], high glucose (HG) could be one of the factors inducing endothelial dysfunction in GDM [11,12]. Indeed, previous studies in HUVECs showed that HG increased the expression of P~Ser¹¹⁷⁷eNOS and hCAT-1, as well as enhanced the transport of L-Arginine and the production of NO [13], suggesting an important role for high glucose on fetoplacental endothelial dysfunction in GDM.

Another mechanism that could be responsible for fetoplacental endothelial dysfunction may be exosomes. An enhanced release of as well as a change in exosomal cargo have recently been implicated in several vascular disorders [14–16]. Exosomes are extracellular nanovesicles, which are released by different cell types, including placental and endothelial cells, cells such as HUVECs [17]. Exosomal cargo can include proteins, mRNAs and microRNAs [18], which are able to modulate endothelial cell function [19]. Different studies have shown that the placental exosomal concentration is increased in maternal blood during pregnancy [20] and that this amount is even higher in pregnancy complications such as GDM [21]. Furthermore, HG seems to increase the release of exosomes by trophoblast cells [21] and is able to modulate the exosomal cargo of endothelial cell exosomes

[17]. However, a role for exosomes in fetoplacental endothelial function has not been demonstrated up to now.

In the present study, we tested the hypothesis that high D-glucose exposure changes exosome composition and subsequently can induce endothelial cell dysfunction in HUVECs and is a possible mechanism for fetal endothelial dysfunction in GDM. To this end, we incubated HUVECs with basal and high levels of D-glucose and isolated the exosomes. These exosomes were incubated with HUVECs after which protein and mRNA levels were studied of hCAT-1, eNOS and P~Ser¹¹⁷⁷eNOS and VEGF. To determine endothelial cell functionality and activation, we performed a wound healing assay and evaluated ICAM-1 expression in HUVECs incubated with exosomes.

2. MATERIALS AND METHODS

2.1 Cell culture and treatment

Human umbilical vein endothelial cells (HUVECs) were provided by the Endothelial Cell facility of the UMCG (Groningen, Netherlands). HUVECs were isolated by collagenase digestion (0.25 mg/mL Collagenase Type II from *Clostridium histolyticum*; Boehringer, Mannheim, Germany), as previously described [22]. HUVECs were cultured in HUVECs culture medium (RPMI (Lonza, Basel, Switzerland) supplemented with 2 mmol/L L-Glutamine, 5 U/mL Heparin, 100 IE/mL Penicillin, 100 µg/mL Streptomycin, 50 µg/mL Crude ECGF solution and 20% FCS) at 37°C, 5% CO₂ on gelatin-precoated tissue culture flasks (Corning® Costar®; Sigma-Aldrich, Zwijndrecht, The Netherlands). The final D-glucose concentration in this medium was 8.8 mmol/L.

Exposure to high glucose: HUVECs in passage 2 with 70% confluence were incubated with exosome-free media for 24 h, with addition of glucose (HUVEC-HG; RPMI medium D-glucose concentration 25 mmol/L (high glucose (HG)) or without addition of D-glucose (HUVEC-BG; RPMI medium glucose concentration 5.5 mmol/L glucose (basal glucose (BG))). Exosome free medium was obtained after ultracentrifugation of HUVECs culture medium (100 000 g at 4°C for 70 min), supernatant was filtered (0.22 µm filter) and pellet was discarded.

2.2 Exosome isolation and purification

Exosomes were isolated according to Théry et al [23]. Supernatant (40 mL) from HUVECs (HG and BG condition) was collected after

24 h of culture. Exosomes were isolated and purified by differential ultracentrifugation. In brief, supernatant was filtered through a 0.22 μm filter and then, centrifugation was performed at 2000 g at 4°C for 30 min, followed by 12 000 g at 4°C for 45 min. Then, the supernatant was centrifuged at 110 000 g at 4°C for 70 min (Centrikon T-1080 ultracentrifuge, Kontron Instruments). The pellet was resuspended in PBS (pH 7.4) and again centrifuged at 110 000 g at 4°C for 70 min. The exosome pellet was resuspended in sucrose/HEPES 2 mol/L and subsequently purified using a discontinuous sucrose gradient (following the protocol described by Théry et al; from 2 mol/L to 0.25 mol/L). Thus, exosomes were mixed with 2 mL of 2 mol/L sucrose in HEPES and placed on the bottom of a SW41 centrifuge tube, overlaid with 1.5 mL 1.35 mol/L sucrose, 0.5 mL 1.28 mol/L sucrose, 1 mL 1.15 mol/L sucrose and 5 mL 0.25 mol/L sucrose, and ultracentrifuged for 16 h at 210 000 g at 4°C. Thus, 12 fractions were collected with 1 mL sucrose and refractive index was measured. After this, all fractions were washed and ultracentrifuged again with PBS at 110 000 g at 4°C for 70 min. Fraction 11 showed floating density for exosomes (1.16 g/mL) and exosomal markers by western blotting and was used for the experiments described below. The final pellet was resuspended in 300 μL of PBS and stored at -80°C for later incubation experiments with HUVECs. The exosomal protein concentration was determined using the BSATM Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA).

2.3 Preparation of Exosomes for TEM

A 10 μL sample of the exosome suspension was allowed to adhere on freshly prepared Formvar coated 200 mesh EM grids for 2 min. After draining the liquid with a filter paper, samples were stained for 1 min on a drop of 2% ammonium molybdate in water. After draining the liquid again with filter paper and airdrying for 5 min, samples were examined in a FEI Cm100 transmission electron microscope operated at 80 KV. Pictures were taken with a Morada camera using Olympus-sis software.

2.4 Nanoparticle tracking analysis

Quantification and size distribution of exosomes were determined using the NTA equipped with a 405 nm laser with LM14 module (NanoSight NTA 3.0 Nanoparticle Tracking, Version 3.0). Samples were diluted with PBS (1/10) prior to analysis in order to obtain particle distribution of particles per image (50 particles per image). Samples were measured using capture 60 and repeat 5. Settings were

kept constant in all samples. Each video was analyzed to get the mean particle size and the concentration of particles.

2.5 Western blotting of exosomes

Exosome pellets isolated from BG and HG HUVECs were lysed with RIPA buffer (1X) (Radio-Immunoprecipitation Assay buffer, 25 mmol/L Tris•HCl pH 7.6, 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) (Pierce®RIPA Buffer, Thermo Fisher Scientific Inc, Landsmeer, Netherlands) at room temperature for 5 min and then loading buffer (5X) was added to the samples and incubated at 95 °C for 5 min. Twenty µg of exosomal protein per well was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (Immobilon®-FL, Millipore, Amsterdam-Zuidoost, Netherlands). Membranes were probed with primary antibody, polyclonal rabbit anti-CD63 (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and monoclonal mouse anti-HSP70 (1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). After incubation at 4°C overnight, membranes were washed in Tris buffered saline (pH 7.4) and incubated for 1 h at room temperature with secondary antibody IRDye® 800CW or 680CW Secondary Antibodies (LI-COR, Lincoln, Nebraska USA). Membranes were analyzed using LI-COR Odyssey Scanner (LI-COR, Lincoln, Nebraska USA).

2.6 Treatment of HUVECs with exosomes

To study the differential effects of exosomes from HUVEC-BG or HG on endothelial cells, exosomes from HUVEC-HG or HUVEC-BG isolated as described above, were incubated in high or basal-glucose HUVECs (Figure 1). Thus, confluent HUVECs in passage 2 were preincubated with medium containing high or basal glucose for 24 h. Then HUVECs cultures were washed and exposed to exosomes from HUVEC-BG or HG in exosome-free medium for another 12 h in two different exosome concentrations: 0.5 µg protein/cm² (exo-BG 5µg for exosomes derived from HUVEC-BG or exo-HG 5µg for exosomes derived from HUVEC-HG, equivalent to ~2x10⁵ particles per cm²) and 0.1 µg protein/cm² (exo-BG 1µg for exosomes derived from BG or exo-HG 1µg for exosomes derived from HG, equivalent to ~4x10⁴ particles per cm²). After the incubation, cells were washed with PBS and prepared for Western blotting or qPCR. HUVEC-BG and HUVEC-HG without exosomes were used as controls.

2.7 Wound healing assay

HUVECs were plated in 6 wells plates (Corning® Costar®; Sigma-Aldrich, Zwijndrecht, The Netherlands). When, 100% confluence was reached, HUVECs were exposed to HG or BG for 24 h and after that, cells were washed with PBS and incubated with exosomes from high or basal-glucose HUVECs as described above (see also Figure 1). After 12 h incubation with exosomes a scratch was made on a uniform confluent layer of HUVECs using a sterile micropipette tip and cells were washed with PBS to remove debris. Photographs (Leica MC 120 HD camera, ©Leica Microsystems, Amsterdam, The Netherlands) of the same area of the wound were taken immediately after the scratch, after 4 h and after 8 h, to measure the area of the wound. The area under the curve, equivalent to wound healing from time 0 to time 8, was measured using ImageJ.

2.8 Western blotting

HUVECs were plated in 6 wells plates (Corning® Costar®; Sigma-Aldrich, Zwijndrecht, The Netherlands). When, 100% confluence was reached, HUVECs were exposed to HG or BG for 24 h and after that, HUVECs were washed with PBS and incubated with exosomes as described above (see also Figure 1). After 12 h incubation HUVECs were washed with PBS and were lysed with 100 µL of RIPA buffer and protein concentration was determined using BSA™ Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA). Proteins (20 µg) per well from different conditions were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes. Membranes were probed with the following primary antibodies, monoclonal mouse anti-total eNOS (eNOS 1:2000; BD Transduction Laboratories™, CA, USA), monoclonal mouse anti-phosphorylated eNOS at serine¹¹⁷⁷ (P~Ser¹¹⁷⁷eNOS 1:1000; BD Transduction Laboratories™, CA, USA), polyclonal rabbit anti-human cationic transporter type 1 (hCAT-1 1:200; abcam®, Cambridge, UK), polyclonal rabbit anti-vascular endothelial growth factor (VEGF 1:200; abcam®, Cambridge, UK), monoclonal mouse anti-β-actin (1:2500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). After incubation at 4°C overnight, membranes were washed in Tris buffered saline (pH 7.4) and incubated for 1 h at room temperature with secondary antibody IRDye® 800CW or 680CW Secondary Antibodies (LI-COR, Lincoln, Nebraska, USA). Membranes were analyzed using LI-COR Odyssey Scanner (LI-COR, Lincoln, Nebraska, USA).

2.9 qPCR

HUVECs were plated in 6 wells plates (Corning® Costar®; Sigma-Aldrich, Zwijndrecht, The Netherlands). When, 100% confluence was reached, HUVECs were exposed to HG or BG for 24 h and after that, HUVECs were washed with PBS and incubated with exosomes as described above (see also Figure 1). After 12 h incubation, total RNA was isolated using a Qiagen RNeasy kit (Qiagen, Crawley, UK), and RNA quality and integrity were confirmed via gel visualization and spectrophotometric analysis ($OD_{260/280}$). RNA aliquots (1 μ g) were reverse-transcribed into cDNA by First-Strand cDNA Synthesis using SuperScript® II Reverse Transcriptase (Invitrogen™ by life technologies™, Thermo Fisher Scientific Inc, Landsmeer, Netherlands). Real time RT-PCR experiments were performed using an MxPro 3000™ thermal cycler (Stratagene, La Jolla, CA, USA) in a reaction mixture containing 0.5 μ mol/L primers and SYBR green qPCR Master Mix (Stratagene). hCAT-1 (hCAT-1 (F): 5'-GAGTTAGATCCAGCAGACCA-3'; hCAT-1 (R): 5'-TGTTCAACAATTAGCCCAGAG-3'), eNOS (eNOS (F): 5'-CCAGCTAGCCAAAGTCACCAT-3'; eNOS (R): 5'-GTCTCGGAGCCATACAGGATT-3') and VEGF (VEGF-A (F): 5'-TGCGGATCAAACCTCACCAA-3'; VEGF-A (R): 5'-TGTCACATACGC TCCAGGACTT-3') [24] expression gene were analyzed. 28S (28S (F): 5'-TTGAAAATCCGGGGGAGAG-3'; 28S antisense (R): 5'-ACATTGTTCCAACATGCCAG-3') was used as reference gene. The Ct value was defined as the PCR cycle number at which the fluorescent signal of the probes exceeded background and was used for calculating gene expression data with the $2^{-\Delta\Delta Ct}$ method.

2.10 Flow cytometry analysis

To measure whether high glucose or exosomes can activate HUVECs, ICAM-1 expression on HUVECs was measured using flow cytometry. Therefore, HUVECs were exposed to culture medium with HG or BG for 24 h, followed by treatment with exosomes from HUVEC-BG and HUVEC-HG as described above (see also Figure 1). After exosomal exposure, HUVECs were washed with PBS, trypsinized and incubated with a solution of PBS-FCS 5% and mouse anti-FITC conjugated anti-ICAM-1 (BioLegend Inc. Uithoorn, The Netherlands). Mouse IgG1 κ (BioLegend Inc. Uithoorn, The Netherlands) was used as isotype control to test for non-specific antibody binding. HUVEC-BG exposed to LPS (lipopolysaccharide, 2 μ g by 4 h at 37°C) served as positive control and were always positive. To measure dead cells (apoptotic cells) propidium iodide staining (PI) was performed. Flow cytometer analysis was performed on a BD FACSCalibur with two lasers (488

and 635 nm) (BD Biosciences, Breda, The Netherlands). ICAM-1 expression in HUVECs was analysed using Kaluza Flow Cytometry Analysis Software. HUVECs were gated based on size and scatter in the forward-side scatter plot. In a forward scatter-PI plot, live HUVECs were selected as cells negative for PI. These live HUVECs were copied to a forwardscatter-FITC plot. The isotype of each sample was used to set a gate excluding all positive cells. This gate was copied to the sample stained for ICAM-1 and percentage positive cells were determined.

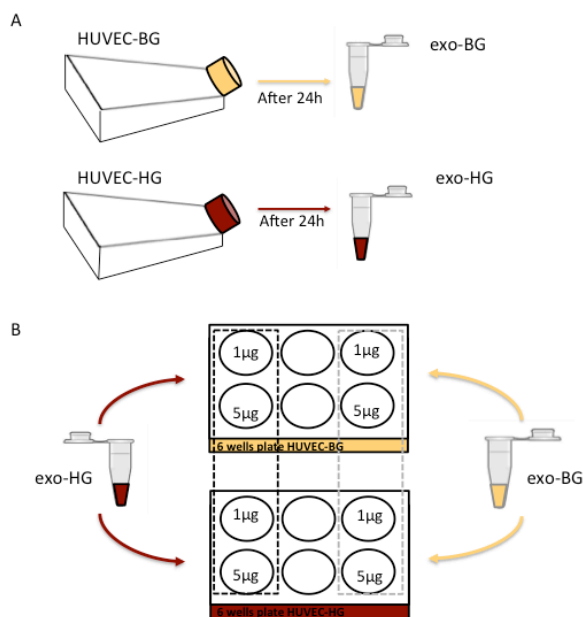


Figure 1: Representative scheme of exosome isolation and exposure. A: exosomes were isolated from the supernatant of HUVECs exposed to basal glucose (HUVEC-BG) and high glucose (HUVEC-HG), after 24 h incubation with exosome-free endothelial culture medium. Exosomes from BG and HG (exo-BG and exo-HG, respectively) were purified and characterized. B: HUVEC-BG and HG were exposed during 12 h with exosomes from both conditions, using 0.1 or 0.5 µg protein/cm² (1 or 5 µg, respectively). HUVEC-BG or HG without exosome treatments were used as controls.

3. RESULTS

3.1 High glucose enhanced exosomal release but reduced the size

Exo-HG and exo-BG were purified and 12 fractions were collected after sucrose gradient isolation. Exosomes were characterized by anti-HSP70 and anti-CD63 staining and were only found in fraction 11 (Figure 2A). This fraction was used for the subsequent experiments. Fractions 11 were studied by electron microscopy and exosomes were

found in all fractions (Figure 2B). NTA showed that exosomes from HUVEC-BG, had a median size of about 135 nm (Figure 2C), which is the expected size for exosomes [23]. HG significantly increased the concentration of exosomes in the supernatant (Figure 2E). The mean size but not the distribution of the different sizes of the exo-HG was similar to the mean size of exo-BG (Figure 2F). Figure 1G, showing particle size distribution, demonstrates a higher concentration of smaller particles in HUVEC-HG and a higher concentration of larger particles in HUVEC-BG.

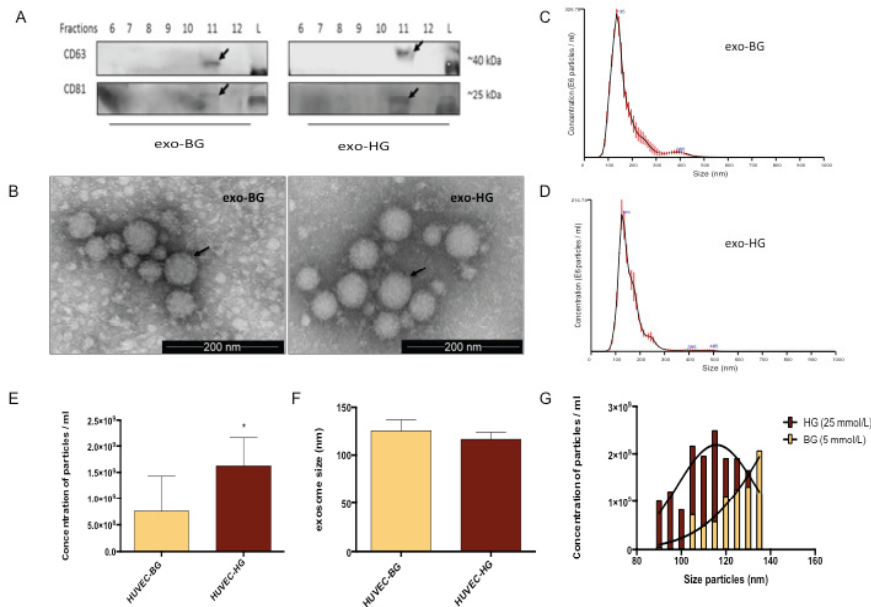


Figure 2: Exosomes derived from HUVECs exposed to basal-glucose (BG) and high glucose (HG). A: exosomes were isolated by the ultracentrifugation protocol. After sucrose gradient, western blotting for each fraction was performed. Only fraction 11 contained exosomes. B: photographs from electronic microscopy showing exosomes in fraction 11 from HUVEC-HG and HUVEC-BG. C and D: Representative graphs for exosomes from fractions 11, using Nanosight (mean=120nm for exosomes derived from high glucose (exo-HG) and mean=135nm for exosomes derived from basal-glucose (exo-BG)). E: median with interquartile range for NTA analysis for all experiments for exosomal concentration (n=7). F-G: particle size and particle size distribution as measured with NTA for all experiments (n=7). * $p < 0.05$, Wilcoxon signed paired test.

3.2 High D-glucose and exosomes derived from HUVECs exposed to high D-glucose increased endothelial cell migration

The exposure of HUVECs to HG increased endothelial migration

compared with HUVEC-BG (Figure 3A, left set of photographs), as also evidenced by a lower area under the curve (Figure 3B). To evaluate whether this effect of HG could be due to change in exosome composition, HUVEC-BG were exposed to exo-HG and HUVEC-HG were exposed to exo-BG. Exo-HG increased endothelial cell migration in HUVEC-BG, and this was significantly higher with HUVEC-BG, but not different from HUVEC-HG (Figure 3B). No changes in HUVEC-HG exposed to exo-BG were observed compared with HUVEC-HG or HUVEC-BG (Figure 3B). Exo-BG did not affect cell migration in HUVEC-BG; exo-HG did not influence cell migration in HUVEC-HG (Figure 3C).

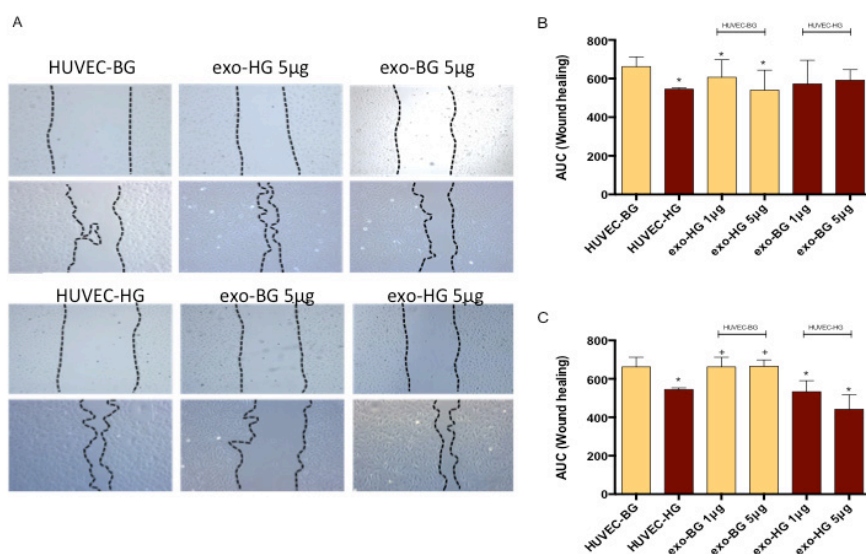


Figure 3: Effect of high glucose and exosomes on endothelial cell migration. HUVEC-BG and HUVEC-HG were incubated with exosomes from BG (exo-BG 1μg or exo-BG 5μg) or from HG (exo-HG 1μg or exo-HG 5μg). A: photographs of the wound healing assay in HUVEC-BG (top photographs) and HUVEC-HG (lower photographs). Area indicated by dotted lines is the wound area. As can be seen, high glucose and exosomes from HUVEC-HG increased the wound healing. Exosomes from BG decreased the wound healing in HUVEC-HG. B: the median with interquartile range of the area under curve of the wound healing from 0-8 h (n=8), $p < 0.05$, Wilcoxon signed rank test, * vs HUVEC-BG, + vs HUVEC-HG.

3.3 High D-glucose and exosomes derived from HUVEC-HG and BG modulated eNOS activation, hCAT-1 and VEGF protein expression in endothelial cells

HG significantly increased the protein expression of P~Ser1177eNOS,

hCAT-1 and VEGF but not of total eNOS (First two columns in Figure 4C, D, E and F, respectively). Exo-HG significantly increased the activation of P~Ser¹¹⁷⁷eNOS in HUVEC-BG. Exo-BG decreased P~Ser¹¹⁷⁷eNOS expression in HUVEC-HG but this was not statistical significant (Figure 4C). No effect on P~Ser¹¹⁷⁷eNOS expression was observed when exo-BG were incubated with HUVEC-BG or when exo-HG were incubated with HUVEC-HG (Figure 4D).

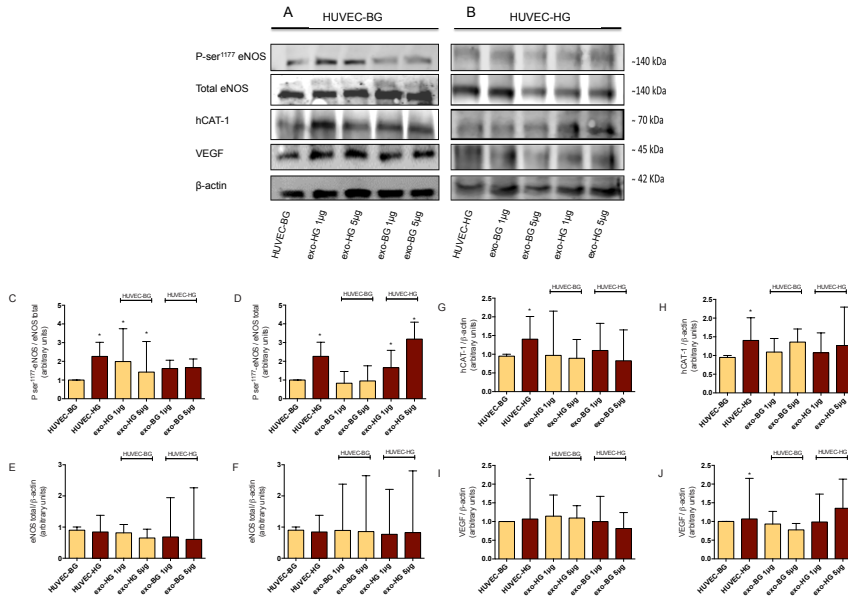


Figure 4: Effect of high glucose and exosomes on eNOS, hCAT-1 and VEGF protein expression by HUVECs. HUVEC-BG and HUVEC-HG were incubated with exosomes from HG (exo-HG 1 μg or exo-HG 5 μg) or from BG (exo-BG 1 μg or exo-BG 5 μg). A and B: representative pictures of Western blots for phosphorylated eNOS (P~Ser¹¹⁷⁷eNOS), total eNOS, hCAT-1 and VEGF. C-J analysis of western blots for phosphorylated eNOS (P~Ser¹¹⁷⁷eNOS), total eNOS, hCAT-1 and VEGF (n=8). * p<0.05 vs HUVEC-BG, Wilcoxon signed rank test.

3.4 High D-glucose and exosomes derived from HUVECs modulated eNOS, hCAT-1 and VEGF gene expression in endothelial cells

The gene expression of eNOS, hCAT-1 and VEGF (first two columns in Figure 5A, C and E, respectively) was higher in HUVEC-HG as compared with HUVEC-BG. eNOS gene expression was not affected in HUVEC-BG exposed to HG-exosomes. eNOS expression in HUVEC-HG was decreased by BG-exosomes only at the lowest concentrations (Figure 5A). Exo-BG did not affect eNOS expression in HUVEC-BG but exo-HG significantly increased eNOS expression

in HUVEC-HG as compared to HUVEC-HG (Figure 5B). hCAT-1 gene expression was not affected by exo-HG incubated with HUVEC-BG or by exo-BG incubated with HUVEC-HG (Figure 5C). Exo-BG (lowest concentration) incubated with HUVEC-BG decreased hCAT-1 mRNA expression as compared with HUVEC-BG. Exo-HG incubated with HUVEC-HG increased hCAT-1 mRNA expression as compared with HUVEC-HG. Exo-HG increased VEGF gene expression in HUVEC-BG (Figure 5E) to levels significantly higher than those in HUVEC-BG and HUVEC-HG. HUVEC-HG was not affected by exo-BG (Figure 5E). Exo-HG increased VEGF gene expression in HUVEC-HG to levels significantly higher than those in HUVEC-HG (Figure 5F).

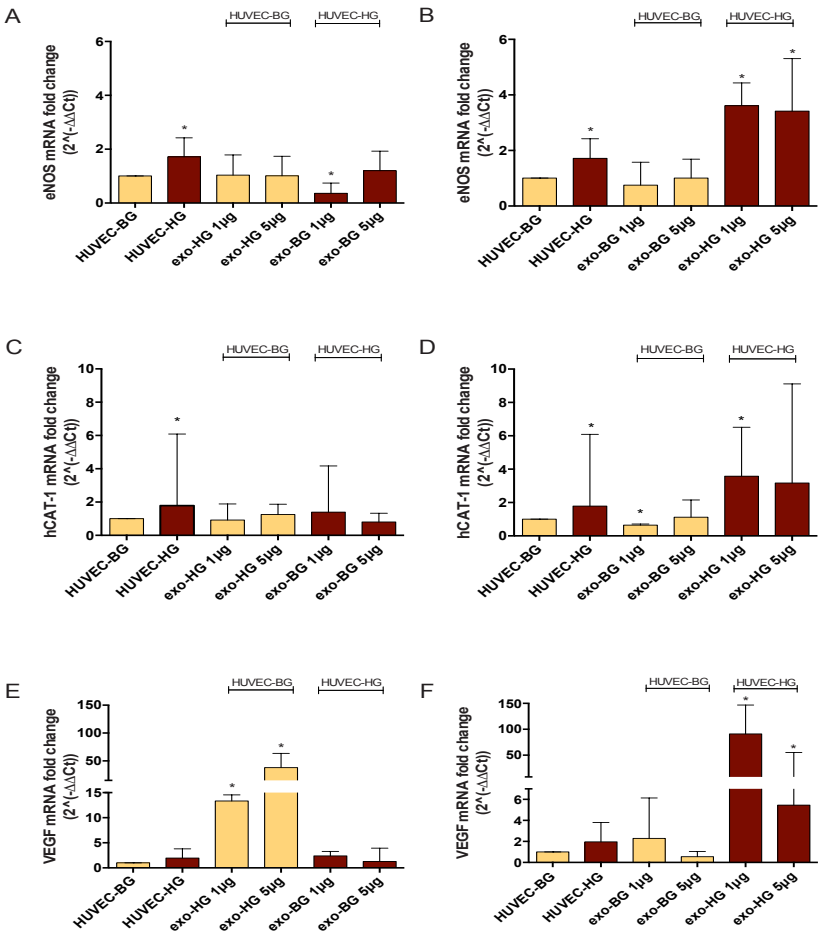


Figure 5: Effect of high glucose and exosomes on eNOS, hCAT-1 and VEGF gene expression by HUVECs. HUVEC-BG and HUVEC-HG were incubated with HG and BG and with exosomes from HG (exo-HG 1μg or exo-HG 5μg) or from BG (exo-BG 1μg or exo-BG 5μg). The effect of high glucose and exosomes was measured on gene expression of phosphorylated eNOS (P-Ser1177eNOS), total eNOS, hCAT-1 and VEGF (n=8), $p < 0.05$, Wilcoxon signed rank test, * vs HUVEC-BG, + vs HUVEC-HG.

3.5 High D-glucose and exosomes derived from HUVECs modulate ICAM-1 expression

HG increased the expression of ICAM-1 in HUVECs (Figure 6A). Exo-HG increased ICAM-1 expression in HUVEC-BG as compared to HUVEC-BG (Figure 6A); however, no changes were observed in HUVEC-HG exposed to exo-B as compared to HUVEC-HG (Figure 6A). No differences in ICAM-1 expression were found in HUVEC-BG exposed to exo-BG and in HUVEC-HG exposed to exo-HG (Figure 6B).

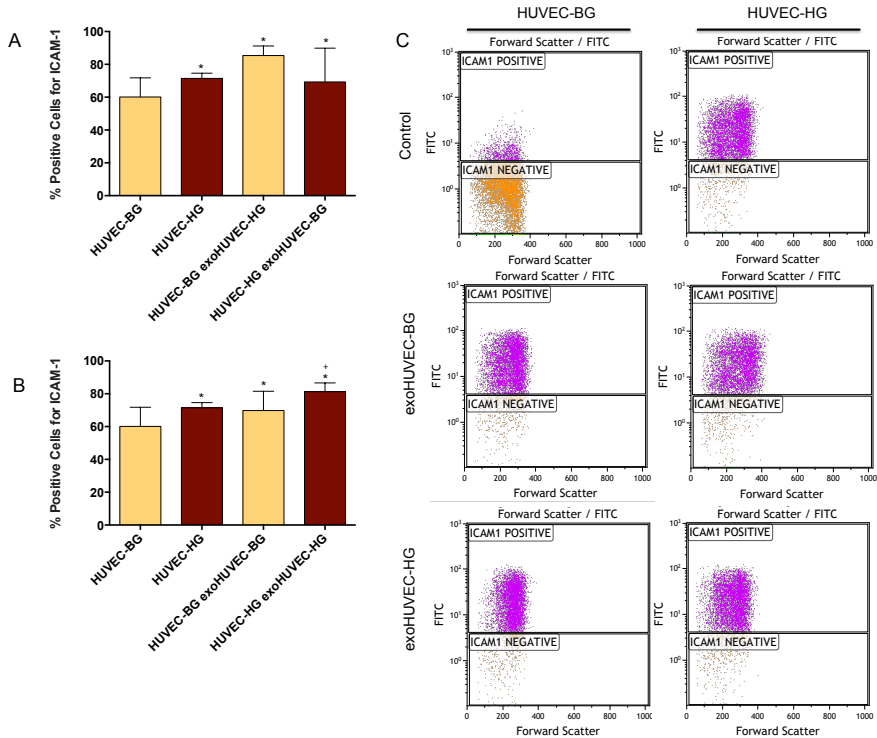


Figure 6: Effect of high glucose and exosomes on ICAM-1 expression by HUVECs. HUVECs were incubated with HG and BG and HUVEC-BG and HUVEC-HG were incubated with exosomes from BG (exo-BG 1 μ g or exo-BG 5 μ g) or from HG (exo-HG 1 μ g or exo-HG 5 μ g). Representative dot plots are shown in C. A and B: percentage ICAM-1 positive cells for the various condition (n=5). $p < 0.05$, Wilcoxon signed rank test, * vs HUVEC-BG, + vs HUVEC-HG.

4. DISCUSSION

Our data confirm our hypothesis that HG can induce endothelial cell dysfunction in HUVECs by changing exosomes. These results showed enhanced endothelial cell migration, a stronger activation of the L-Arginine/NO signaling pathway and increased endothelial cell activation. The resulting endothelial phenotype has many similarities

with the phenotype of HUVECs from GDM [24].

This study shows that incubation of HUVECs with HG increased the release of exosomes. This finding does not corroborate the findings of de Jong et al [17], who was not able to demonstrate an effect of hyperglycemia on exosomal release from human microvascular endothelial cells [17]. This might be explained by differences in the experimental setup such as application of an adult endothelial cell type instead of an immature neonatal and a lower glucose concentration (18 mmol/L vs 25 mmol/L). Our results are however in line with the findings of Rice et al showing in trophoblasts that hyperglycemia increased the release of exosomes [19]. In our study, HG not only enhanced the exosomal number but also modulated the exosomal size. Although no differences were found in mean exosomal size between BG and HG, HG modified the distribution pattern of the exosomal size, causing higher concentrations of smaller exosomal particles than BG. Interestingly, also higher concentration of exosomes are found in maternal blood from women diagnosed with GDM [21].

We found in HUVEC-HG an increased endothelial cell migration compared to HUVEC-BG. This is in line with other studies [25,26] in which it was observed that exposure to HG induced an expedited endothelial cell migration. A possible mechanism for this is the observed enhanced production of VEGF by HUVECs when exposed to HG [27]. Interestingly, HUVECs isolated from GDM pregnancies grew slower than HUVECs from healthy pregnancy, despite increased levels of VEGF [8].

Interestingly, our results demonstrate that only exo-HG increased endothelial cell migration and not exo-BG, indicating that the effect of HG on endothelial cell migrations is mediated by exosomes. In fact, exo-BG incubated with HUVEC-HG were able to restore endothelial cell migration to normal values. The increased endothelial cell migration after incubation with exo-HG was associated with an upregulation of VEGF production. This is corroborating the findings of Li et al, who showed that exosomes from endothelial progenitor cells can increase endothelial cell migration in a wound healing assay with a human microvascular endothelial cell line [28]. Similar to our study, Li, et al also showed that the exosomes upregulated proangiogenic molecules such as VEGF [28].

We previously found endothelial cell dysfunction in HUVECs from patients with GDM [9]. This dysfunction was characterized by increased P~Ser¹¹⁷⁷eNOS and hCAT-1 expression. The present study shows that HG also increased the expression of P~Ser¹¹⁷⁷eNOS, hCAT-

1 and VEGF (the latter also at the mRNA level) in HUVECs. Similar data have been shown by Vasquez [13], who found that exposure of HUVECs to HG increased the expression of P~Ser¹¹⁷⁷eNOS and hCAT-1. They also showed increased L-Arginine transport and NO synthesis. We show that most of the effects of HG on HUVECs can also be induced by exo-HG in HUVECs incubated under BG conditions. We found that exo-HG increased P~Ser¹¹⁷⁷eNOS, hCAT1 and VEGF expression, although this was only significant for P~Ser¹¹⁷⁷eNOS expression. Interestingly, exo-BG can restore the values of P~Ser¹¹⁷⁷eNOS in HUVEC-HG to normal values. Exo-BG also seemed to be able to restore the endothelial expression of hCAT-1 and VEGF, although this was not statistical significant. These results show that exo-HG can induce a HG phenotype in HUVEC-BG, and are therefore in line with the hypothesis that HG induces the production of exosomes in fetal endothelium and that these exosomes negatively impact fetal endothelial function.

In our study, endothelial cells incubated under HG conditions showed increased endothelial cell activation, as quantified by the expression of ICAM-1. Previous studies have also shown that HG can activate endothelial cells by increasing expression of this adhesion molecule [29,30]. Since HUVECs from GDM pregnancies also express high levels of ICAM-1 [31], HG may be one of the responsible factors for fetoplacental endothelial cell activation in GDM. Moreover, the expression of ICAM-1 is lower in HUVECs from women with GDM with HbA1c levels lower than 6% as compared with women with normal pregnancies, suggesting these differences can be due to differences in glucose management [32]. Although we found that this upregulation of ICAM-1 could also be induced by exo-HG, which is in line with our hypothesis, endothelial cell activation was also induced by exo-BG. In summary, our results indicate that HG induced endothelial cell dysfunction and activation HUVECs, which was similar to endothelial cell dysfunction and activation in HUVECs from GDM. We also show that HUVEC exosomes have the capacity to modulate endothelial function in HUVECs, either under BG or HG conditions. Although incubation of HUVECs with HG mimicked many of the features of endothelial cell dysfunction in the HUVECs from GDM, the exposure HUVECs to cannot explain the total phenotype GDM HUVECs. This may suggest that other factors, such as high insulin levels or proinflammatory factors, may also play a role. Furthermore, our data suggests that fetoplacental endothelial exosomes are one of the mechanisms behind endothelial dysfunction induced by HG. Therefore, we hypothesize that fetoplacental exosomes can also be

involved in fetoplacental endothelial dysfunction in GDM.

REFERENCES

1. American Diabetes Association (ADA), Standard of medical care in diabetes - 2017, *Diabetes Care*. 40 (sup 1) (2017) s4–s128. doi:10.2337/dc17-S001.
2. B.S. Buckley, J. Harreiter, P. Damm, R. Corcoy, A. Chico, D. Simmons, A. Vellinga, F. Dunne, Gestational diabetes mellitus in Europe: Prevalence, current screening practice and barriers to screening. A review, *Diabet. Med.* 29 (2012) 844–854. doi:10.1111/j.1464-5491.2011.03541.x.
3. Hedderson, Gestational diabetes mellitus and lesser degrees of pregnancy hyperglycemia: association with increased risk of spontaneous preterm birth, *Obstet. Gynecol.* 102 (2003) 850–856. doi:10.1016/S0029-7844(03)00661-6.
4. Nordin, J.W.H. Wei, N.N. Naing, E.M. Symonds, Comparison of maternal-fetal outcomes in gestational diabetes and lesser degrees of glucose intolerance+, *J. Obstet. Gynaecol. Res.* 32 (2006) 107–114. doi:10.1111/j.1447-0756.2006.00360.x.
5. Y.Y. Hirsch L, Impact of gestational hyperglycemia on maternal and child health., *Curr. Opin. Clin. Nutr. Metab. Care.* 17 (2014) 255–260. doi:10.1097/MCO.000000000000030.
6. C.H. Krishnaveni, Ghattu V; Veena, Sargoor R; Jones, Alexander; Srinivasan, Krishnamachari; Osmond, Clive; Karat, Samuel C; Kurpad, Anura V; Fall, Exposure to maternal gestational diabetes is associated with higher cardiovascular responses to stress in adolescent Indians, *J. Clin. Endocrinol. Metab.* 100 (2014) 986–996. doi:https://doi.org/10.1210/jc.2014-3239.
7. J.H. Moon JH, Kwak SH, Prevention of type 2 diabetes mellitus in women with previous gestational diabetes mellitus FAU - Moon, Joon Ho FAU - Kwak, Soo Heon FAU - Jang, Hak C., *Korean J Intern Med.* 32 (2017) 26–41. doi:10.3904/kjim.2016.203.
8. S.A. Sultan, W. Liu, Y. Peng, W. Roberts, D. Whitelaw, A.M. Graham, The Role of Maternal Gestational Diabetes in Inducing Fetal Endothelial Dysfunction, *J. Cell. Physiol.* 230 (2015) 2695–2705. doi:10.1002/jcp.24993.
9. F. Westermeier, C. Salomón, M. González, C. Puebla, E. Guzmán-Gutiérrez, F. Cifuentes, A. Leiva, P. Casanello, L. Sobrevia, Insulin Restores Gestational Diabetes Mellitus–Reduced Adenosine Transport Involving Differential Expression of Insulin Receptor Isoforms in Human Umbilical Vein Endothelium, *Diabetes.* 60 (2011) 1677 LP-1687. doi:10.2337/db11-0155.
10. A. Leiva, B. Fuenzalida, E. Barros, B. Sobrevia, R. Salsoso, T. Sáez, R. Villalobos, L. Silva, I. Chiarello, F. Toledo, J. Gutiérrez, C. Sanhueza, F.P. and L. Sobrevia, Nitric Oxide is a Central Common Metabolite in Vascular Dysfunction Associated with Diseases of Human Pregnancy, *Curr. Vasc. Pharmacol.* 14 (2016) 237–259. doi:http://dx.doi.org/10.2174/1570161114666160222115158.
11. E.A. Reece, G. Leguizamón, A. Wiznitzer, Gestational diabetes: the need for a common ground, *Lancet.* 373 (2017) 1789–1797. doi:10.1016/S0140-6736(09)60515-8.
12. B.E. METZGER, Long-term Outcomes in Mothers Diagnosed With Gestational Diabetes

- Mellitus and Their Offspring, *Clin. Obstet. Gynecol.* 50 (2007). http://journals.lww.com/clinicalobgyn/Fulltext/2007/12000/Long_term_Outcomes_in_Mothers_Diagnosed_With.15.aspx.
13. R. Vásquez, M. Fariás, J.L. Vega, R.S. Martin, A. Vecchiola, P. Casanello, L. Sobrevia, D-glucose stimulation of L-arginine transport and nitric oxide synthesis results from activation of mitogen-activated protein kinases p42/44 and Smad2 requiring functional type II TGF- β receptors in human umbilical vein endothelium, *J. Cell. Physiol.* 212 (2007) 626–632. doi:10.1002/jcp.21057.
 14. H.J. Huber, P. Holvoet, Exosomes: emerging roles in communication between blood cells and vascular tissues during atherosclerosis, *Curr. Opin. Lipidol.* 26 (2015). http://journals.lww.com/co-lipidology/Fulltext/2015/10000/Exosomes____emerging_roles_in_communication_between.10.aspx.
 15. A. Tokarz, I. Szuścik, B. Kuśnierz-cabala, M. Kapusta, A. Georgescu, E. Stępień, Extracellular vesicles participate in the transport of cytokines and angiogenic factors in diabetic patients with ocular complications. *PubMed Commons, Folia Med. Cracov.* 4 (2015) 35–48.
 16. Y. Chen, Y. Song, J. Huang, M. Qu, Y. Zhang, J. Geng, Z. Zhang, J. Liu, G.-Y. Yang, Increased Circulating Exosomal miRNA-223 Is Associated with Acute Ischemic Stroke, *Front. Neurol.* 8 (2017) 57. <http://journal.frontiersin.org/article/10.3389/fneur.2017.00057>.
 17. O.G. de Jong, M.C. Verhaar, Y. Chen, P. Vader, H. Gremmels, G. Posthuma, R.M. Schiffelers, M. Gucek, B.W.M. van Balkom, Cellular stress conditions are reflected in the protein and RNA content of endothelial cell-derived exosomes, *J. Extracell. Vesicles.* 1 (2012) 1–12. doi:10.3402/jev.v1i0.18396.
 18. J. Kowal, M. Tkach, C. Théry, Biogenesis and secretion of exosomes, *Curr. Opin. Cell Biol.* 29 (2014) 116–125. doi:10.1016/j.ceb.2014.05.004.
 19. G.E. Rice, K. Scholz-Romero, E. Sweeney, H. Peiris, M. Kobayashi, G. Duncombe, M.D. Mitchell, C. Salomon, The effect of glucose on the release and bioactivity of exosomes from first trimester trophoblast cells, *J. Clin. Endocrinol. Metab.* 100 (2015) E1280–E1288. doi:10.1210/jc.2015-2270.
 20. A. Sabapatha, C. Gercel-Taylor, D.D. Taylor, Specific Isolation of Placenta-Derived Exosomes from the Circulation of Pregnant Women and Their Immunoregulatory Consequences, *Am. J. Reprod. Immunol.* 56 (2006) 345–355. doi:10.1111/j.1600-0897.2006.00435.x.
 21. C. Salomon, K. Scholz-Romero, S. Sarker, E. Sweeney, M. Kobayashi, P. Correa, S. Longo, G. Duncombe, M.D. Mitchell, G.E. Rice, S.E. Illanes, Gestational Diabetes Mellitus Is Associated With Changes in the Concentration and Bioactivity of Placenta-Derived Exosomes in Maternal Circulation Across Gestation, *Diabetes.* 65 (2016) 598 LP-609. <http://diabetes.diabetesjournals.org/content/65/3/598.abstract>.
 22. M.M. Faas, M.G. van Pampus, Z.A. Anninga, J. Salomons, I.M. Westra, R.B. Donker, J.G. Aarnoudse, P. de Vos, Plasma from preeclamptic women activates endothelial cells via monocyte activation in vitro, *J. Reprod. Immunol.* 87 (2010) 28–38. doi:10.1016/j.jri.2010.07.005.
 23. C. Théry, S. Amigorena, G. Raposo, A. Clayton, Isolation and Characterization of

Exosomes from Cell Culture Supernatants and Biological Fluids, *Curr. Protoc. Cell Biol.* (2006). doi:10.1002/0471143030.cb0322s30.

24. Q. Meng, L. Shao, X. Luo, Y. Mu, W. Xu, L. Gao, H. Xu, Y. Cui, Expressions of VEGF-A and VEGFR-2 in placentae from GDM pregnancies, *Reprod. Biol. Endocrinol.* 14 (2016) 61. doi:10.1186/s12958-016-0191-8.
25. T. Sáez, R. Salsoso, C. Sanhueza, F. Pardo, A. Leiva, M. Faas, L. Sobrevia, Gestational diabetes and feto-placental endothelial dysfunction: Role of exosomes from human umbilical vein endothelial cells on L-arginine/NO signalling pathway, *Placenta.* 45 (2017) 111. doi:10.1016/j.placenta.2016.06.173.
26. Q. Huang, N. Sheibani, High glucose promotes retinal endothelial cell migration through activation of Src, PI3K/Akt1/eNOS, and ERKs, *Am. J. Physiol. - Cell Physiol.* 295 (2008) C1647 LP-C1657. <http://ajpcell.physiology.org/content/295/6/C1647.abstract>.
27. M. Farnoodian, C. Halbach, C. Slinger, B.R. Pattnaik, C.M. Sorenson, N. Sheibani, High glucose promotes the migration of retinal pigment epithelial cells through increased oxidative stress and PEDF expression, *Am. J. Physiol. - Cell Physiol.* 311 (2016) C418 LP-C436. <http://ajpcell.physiology.org/content/311/3/C418.abstract>.
28. Y. Shizukuda, S. Tang, R. Yokota, J.A. Ware, Vascular Endothelial Growth Factor–Induced Endothelial Cell Migration and Proliferation Depend on a Nitric Oxide–Mediated Decrease in Protein Kinase Cδ Activity, *Circ. Res.* 85 (1999) 247 LP-256. <http://circres.ahajournals.org/content/85/3/247.abstract>.
29. X. Li, C. Chen, L. Wei, Q. Li, X. Niu, Y. Xu, Y. Wang, J. Zhao, Exosomes derived from endothelial progenitor cells attenuate vascular repair and accelerate reendothelialization by enhancing endothelial function, *Cytotherapy.* 18 (2017) 253–262. doi:10.1016/j.jcyt.2015.11.009.
30. H. Taki, A. Kashiwagi, Y. Tanaka, K. Horiike, Expression of intercellular adhesion molecules 1 (ICAM-1) via an osmotic effect in human umbilical vein endothelial cells exposed to high glucose medium, *Life Sci.* 58 (1996) 1713–1721. doi:10.1016/0024-3205(96)00151-8.
31. L. Piconi, L. Quagliaro, R. Da Ros, R. Assaloni, D. Giugliano, K. Esposito, C. Szabo, A. Ceriello, Intermittent high glucose enhances ICAM-1, VCAM-1, E-selectin and interleukin-6 expression in human umbilical endothelial cells in culture: the role of poly(ADP-ribose) polymerase, *J. Thromb. Haemost.* 2 (2004) 1453–1459. doi:10.1111/j.1538-7836.2004.00835.x.
32. P. Di Fulvio, A. Pandolfi, G. Formoso, S. Di Silvestre, P. Di Tomo, A. Giardinelli, A. De Marco, N. Di Pietro, M. Taraborrelli, S. Sancilio, R. Di Pietro, M. Piantelli, A. Consoli, Features of endothelial dysfunction in umbilical cord vessels of women with gestational diabetes, *Nutr. Metab. Cardiovasc. Dis.* 24 (2017) 1337–1345. doi:10.1016/j.numecd.2014.06.005.
33. M. Kurt, E. Zulfikaroglu, N.L. Ucankus, S. Omeroglu, U. Ozcan, Expression of intercellular adhesion molecule-1 in umbilical and placental vascular tissue of gestational diabetic and normal pregnancies, *Arch. Gynecol. Obstet.* 281 (2009) 71. doi:10.1007/s00404-009-1066-4.